Development 129, 2195-2207 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV3602

Hes6 regulates myogenic differentiation

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Accepted 12 February 2002

SUMMARY

Hes6 is a basic helix-loop-helix transcription factor homologous to *Drosophila* Enhancer of Split (EoS) proteins. It is known to promote neural differentiation and to bind to Hes1, a related protein that is part of the Notch signalling pathway, affecting Hes1-regulated transcription. We show that *Hes6* is expressed in the murine embryonic myotome and is induced on C2C12 myoblast differentiation in vitro. Hes6 binds DNA containing the Enhancer of Split E box (ESE) motif, the preferred binding site of *Drosophila* EoS proteins, and represses transcription of an ESE box reporter. When overexpressed in C2C12 cells, Hes6 impairs normal differentiation, causing a decrease in the induction of the cyclin-dependent kinase inhibitor, $p21^{Cip1}$, and an increase in the number of cells that can be recruited back into the cell cycle after differentiation in culture. In *Xenopus* embryos, *Hes6* is co-expressed with MyoD in early myogenic development. Microinjection of Hes6 RNA in vivo in *Xenopus* embryos results in an expansion of the myotome, but suppression of terminal muscle differentiation and disruption of somite formation at the tailbud stage. Analysis of Hes6 mutants indicates that the DNA-binding activity of Hes6 is not essential for its myogenic phenotype, but that protein-protein interactions are. Thus, we demonstrate a novel role for Hes6 in multiple stages of muscle formation.

Key words: Muscle, Notch, Enhancer of split, Mouse, *Xenopus*

INTRODUCTION

The basic helix-loop-helix (bHLH) family of transcription factors plays a key role in the sequence of cell fate decisions that occurs during differentiation. One group of these proteins are the EoS genes in *Drosophila* (Delidakis and Artavanis-Tsakonas, 1992). EoS genes are expressed after activation of the Notch pathway that regulates cell fate decision and boundary formation (Jennings et al., 1994). EoS proteins act as repressors of transcription and regulate differentiation and cell fate in such diverse contexts as neurogenesis, sex determination and imaginal disc formation. In many but not all cases, the phenotype resulting from EoS overexpression parallels the phenotype produced by Notch activation (Dawson et al., 1995; de Celis et al., 1996; Nakao and Campos-Ortega, 1996).

The vertebrate homologues of EoS have been named *Hes*, *her* and *ESR* genes in mammals, zebrafish and *Xenopus* respectively. These proteins share three highly conserved features. The basic region of the bHLH domain contains a characteristic proline residue. C-terminal to the bHLH domain is a region known as the orange domain, which confers specificity of function to different family members, enhances protein-protein interactions between Hes proteins and has a role in transcriptional repression (Castella et al., 2000; Dawson et al., 1995; Giebel and Campos-Ortega, 1997; Leimeister et al., 2000). At the N terminus is a WRPW motif that binds the transcriptional repressor Groucho and its mammalian homologues, the TLE proteins (Grbavec and Stifani, 1996; Paroush et al., 1994). EoS homologues have a wide range of biological functions in vertebrates, including the regulation of neural development in response to Notch signalling and somite formation (Jen et al., 1999; Ohtsuka et al., 1999).

We are interested in the role of Hes genes in myogenesis. In *Drosophila*, the EoS mutant displays increased numbers of myogenic cells (Corbin et al., 1991). In vitro, overexpression of *Hes1* blocks myogenesis induced by MyoD in 10T1/2 cells (Sasai et al., 1992). *Hes1* transcription is rapidly induced by Notch activation in C2C12 myoblasts, correlating with a block in the ability of cells to differentiate in low serum medium (Jarriault et al., 1998; Kuroda et al., 1999). However, Hes1 overexpression alone does not block C2C12 differentiation, suggesting that other factors are required in this system (Shawber et al., 1996). From this data it seemed likely that other Hes family members might be involved in regulating myogenesis, in addition to *Hes1*. By searching the expressed sequence tag (EST) database, we have identified a novel Hes cDNA in EST clones derived from skeletal muscle libraries. This cDNA has been independently identified by other groups and named Hes6 (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Vasiliauskas and Stern, 2000).

Hes6 shares the key features of EoS proteins described above. The bHLH domain contains the conserved proline residue, the orange domain is present and a WRPW motif is found at the C terminus. A feature that distinguishes *Hes6* from other EoS homologues is the structure of the loop region of the bHLH domain, which is four or five amino acids shorter than that of other Hes proteins (Bae et al., 2000; Koyano-Nakagawa et al., 2000). Surprisingly, Hes6 does not bind to the E or N box motifs recognised by other Hes proteins, leading to the suggestion that it may not bind DNA at all, but rather act by heteromultimerising with other Hes proteins to modify transcription. In support of this, Hes6 binds to mouse Hes1 and XHairy 2A proteins in in vitro assays, and inhibits the transcriptional repressor activity of Hes1 at an N box reporter in co-transfection experiments (Bae et al., 2000; Koyano-Nakagawa et al., 2000).

The function of Hes6 has been investigated in vitro in explant cultures of retinal precursors, where retroviral overexpression of Hes6 promotes rod photoreceptor differentiation at the expense of other cell types (Bae et al., 2000). In *Xenopus*, Hes6 overexpression promotes neural differentiation (Koyano-Nakagawa et al., 2000). Hes6 transcription does not appear to be regulated by Notch activation in *Xenopus* embryos (Koyano-Nakagawa et al., 2000). However, overexpression of the proneural proteins *Xngn1* and *Xash3* induces Hes6, and Hes6 is lost in *Ngn1* knockout mice, indicating that Hes6 transcription may be regulated by these proteins in the developing nervous system. Mice with a homozygous deletion of Hes6 appear phenotypically normal, suggesting that mouse Hes6 is functionally redundant.

Although Hes6 is highly expressed in the developing myotome, its function in myogenesis has not been investigated previously (Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Vasiliauskas and Stern, 2000). We find that Hes6 binds to the ESE box motif that is the preferred binding site of *Drosophila* EoS proteins. We investigated the effects of Hes6 overexpression in C2C12 myoblasts and found that differentiation was inhibited, with a substantial reduction in the proportion of cells undergoing cell cycle withdrawal. In *Xenopus* embryos, Hes6 is also found in myogenic precursors. When overexpressed, it perturbed myogenesis, increasing the size of the myotome but decreasing the expression of a late marker of terminal muscle differentiation. In addition, Hes6 overexpression disrupted somite formation. There was no apparent difference in the phenotype produced by wild-type Hes6 and a mutant that lacked DNA-binding activity in the in vitro or in vivo studies. This suggests that Hes6 mediates its effects by protein-protein interactions, rather than by acting as a transcription factor.

MATERIALS AND METHODS

Plasmids and cloning

Murine Hes6 was identified by searches of the EST database using the tBLASTn programme to identify EST clones encoding proteins homologous to *Drosophila* EoS proteins. A full-length image clone, W66929 was found and was completely sequenced. The sequence was found to be identical to recently reported sequence for Hes6 (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Vasiliauskas and Stern, 2000). An *Eco*RI *Sac*I fragment of murine Hes6 containing the coding region was subcloned into pGEM-3 (Promega), with and without an N-terminal HIS tag, and into pIRES2-EGFP (Clontech). A mutant of murine Hes6, Hes6DBM, in which the basic region amino acids were mutated from VEKKRRARIN to VKEEEDAEIN was created using a Quikchange mutagenesis kit (Stratagene). Murine Hes6 and Hes6DBM were also cloned into pCS2, and into pBabepuro-GFP, a modified pBabepuro vector containing the internal ribosome entry site and the enhanced green fluorescent protein (EGFP) cDNA from pIRES2-EGFP (Clontech) (Morgenstern and Land, 1991).

Xenopus cDNAs encoding wild-type and mutant Hes6 in pCS2 were a gift from Chris Kintner, Salk Institute.

Reporter vectors used were pCOLluc3, comprising the base pairs –520 to +63 of the collagenase promoter upstream of firefly luciferase (a gift from Anna-Lisa Montesanti, Oxford, UK) (Angel et al., 1987), pCOLluc3-ESE, containing two ESE box boxes cloned 5′ of the collagenase promoter, and pRLTK (Promega), a transfection efficiency control.

All PCR generated constructs were verified by complete sequencing, other constructs were sequenced at vector-insert junctions.

Cell culture

C2C12 cells were obtained from the European Collection of Cell Cultures, Salisbury, UK. COS and 3T3 cells were obtained from the American Tissue Culture Collection. GP+E retroviral producer cells were a gift from Dr F. Watt, Imperial Cancer Research Fund, London, UK. GP+E, COS7 and 3T3 cells were maintained in DMEM supplemented with 10% foetal calf serum (FCS; Gibco BRL). C2C12 cells were maintained in growth medium, GM, consisting of DMEM with 15% FCS. For differentiation experiments, C2C12 cells were grown to confluence and then transferred to differentiation medium, (DM), consisting of DMEM plus 2% FCS (Jarriault et al., 1998).

Immunofluorescent staining of C2C12 myoblasts

Primary antibodies used were goat anti-$p21^{Cip1}$ (C 19, Santa Cruz Biotechnology), mouse anti-Troponin-T (clone JLT-12, Sigma), mouse anti-5-bromo-2′-deoxyuridine (BrdU, Dako). Secondary Cy3-conjugated Goat anti-mouse and donkey anti-goat antibodies were from Jackson ImmunoResearch.

Cells were washed in phosphate buffered saline (PBS), fixed in 1% paraformaldehyde (for visualising EGFP) or cold methanol (for immunofluorescence), washed in PBS and blocked in PBS containing 2% serum of the same species as the secondary antibody for 1 hour. Antibodies were diluted in 2% serum. After mounting in Vectashield aqueous mountant with DAPI (Vector Laboratories), cells were photographed on a Zeiss Axiophot II microscope.

To determine the number of $p21^{Cip1}$-positive nuclei, DAPI- and Cy3-stained images were merged using Adobe Photoshop and at least 1000 nuclei from randomly selected fields were counted for each cell type after 48 hours and 120 hours in DM in each experiment.

BrdU labelling

After differentiation for 5 days in DM, C2C12 myoblasts were incubated for 20-22 hours in GM containing 50 μM BrdU, washed in PBS, fixed in cold methanol and then stained for BrdU as described (Celis and Madsen, 1994). DAPI- and Cy3-stained images were merged and at least 1000 nuclei from randomly selected fields were counted for each cell type in each experiment.

Northern blotting

RNA was isolated from C2C12 cells using RNAzol B. An *Eco*RI/*Sac*I fragment of Hes6 cDNA was used as a probe. The myogenin probe was an *Eco*RI, *Not*I fragment of image clone 425649, which was sequenced to confirm it encoded murine myogenin. A mouse multiple tissue northern blot was hybridised according to the manufacturer's instructions (Clontech).

In situ hybridisation of mouse embryos

^{35}S-labelled in situ hybridisation was performed on paraffin-wax embedded sections of mouse embryos by the Imperial Cancer Research Fund in situ hybridisation service as described (Decimo et al., 1993).

Reporter assays

Six-well tissue culture plates (Falcon) were seeded with 10^5 COS or 3T3 cells on the day before transfection. Expression vector (1.66 μg; pIRES2-EGFP-βGal or pIRES2-EGFP–Hes6), 66 ng of *Renilla* luciferase control vector (pRLTK) and 0.33 μg Firefly luciferase reporter vector (pCOLluc3 or pCOLluc3-ESE) were transfected into each well using Superfect (Qiagen) according to the manufacturer's instructions. All transfections were performed in triplicate or quadruplicate wells. Forty-eight hours after transfection cells were lysed and analysed for Firefly and *Renilla* luciferase activity using the dual luciferase reporter system (Promega).

Electrophoretic mobility shift assays (EMSA)

Wild type or N-terminal His tagged murine Hes6 protein was generated using a TnT T7 in vitro transcription and translation kit (Promega). The presence of full-length protein was confirmed by autoradiography of an ^{35}S-methionine labelled translation performed in parallel (data not shown).

The following double stranded oligonucleotides were used in EMSA: ESE box, containing two Enhancer of Split E box (ESE) motifs (underlined), 5′GGTGGCACGTGCCATTTGGCACGTGCC-ATG 3′; E box, containing two E boxes (underlined), 5′GGACACGTGTTCACGTGACATG 3′; N Box, containing two N boxes (underlined), 5′ ACGCCACGAGCCACAAGGATTG 3′ (Jennings et al., 1999). Double stranded oligonucleotides were labelled with ^{32}P by kinase treatment. Labelled oligonucleotide (25 fM) was incubated with 5 μl in vitro translated protein for 45 minutes on ice in a buffer containing 20 mM Hepes pH 7.4, 50 mM KCl, 2 mM β-mercaptoethanol, 10% glycerol, with 0.05 mg/ml poly(dIdC) (Amersham Pharmacia Biotech), and 0.5% bovine serum albumin. In some experiments antibodies were added, either 1 μl of control IgG (Sigma) or 1 μl of anti-HIS antibody (Sigma).

Retroviral expression of mHes6

GP+E cells were transfected using superfect (Qiagen). After selection in 2.5 μg/ml puromycin for 7 days, infected GP+E cells were sorted by flow cytometry to obtain the highest 20% of green fluorescent cells. Supernatants of these cells were used to infect C2C12 myoblasts, by incubation of the conditioned medium in the presence of 8 μg/ml polybrene (Sigma). The infected cells were used in experiments after 7-10 days of puromycin selection. To assess the proportion of cells expressing EGFP, wild-type C2C12 cells (negative control) and infected myoblasts were analysed for EGFP fluorescence by flow cytometry. Cells grown in GM were trypsinised, washed in GM and in PBS, and then fixed in 1% paraformaldehyde; those with green channel fluorescence in excess of the wild-type C2C12 cells were scored as positive.

Xenopus embryos, fixation, β-galactosidase staining and mRNA injection

Xenopus laevis embryos were obtained by hormone induced laying and in vitro fertilisation using standard methods. The embryos were dejellied in 2% cysteine pH 7.8-8.0 then washed and incubated in 0.1× MBS. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Embryo fixation and staining for β-galactosidase (200-300 pg injected per embryo) was performed as described (Sive et al., 2000).

Capped RNAs were synthesised in vitro from nuc-β-gal (Chitnis et al., 1995) XHes6, XHes6DBM, XHes6ΔWRPW (Koyano-Nakagawa et al., 2000), murine Hes6 and Hes6DBM using the SP6 Message Machine kit (Ambion). Where appropriate, embryos were injected in 0.2× MBS supplemented with 6% Ficoll and transferred to 0.1× MBS after gastrulation.

Xenopus embryo in situ hybridisation and antibody staining

Whole mount in situ hybridisation was performed as described (Shimamura et al., 1994). Linearised Bluescript plasmids from Nβtubulin (*Bam*HI/T3), XMyoD (*Hin*dIII/T7) and muscle actin (*Hin*dIII/T7) were used to generate digoxigenin-11-UTP-labeled (Boehringer Mannheim) antisense RNA probes from the indicated polymerases. BM Purple (Boehringer Mannheim) was used as a substrate.

Whole-mount antibody staining was performed as described in the Cold Spring Harbor *Xenopus* Laboratory Manual (2000) using the 12/101 antibody (obtained from DSHB) (Kintner and Brockes, 1984), at 1:500, and an alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) at 1:1000. Antibody staining was developed using NBT and BCIP as colour substrates.

For histological analysis, embryos were embedded in paraffin and sectioned at 10 μm. Monoclonal anti-α-sarcomeric actin clone (5C5) (Sigma) applied at 1:400 for 2 hours at room temperature was recognised with a secondary goat anti-mouse IgM Cy3 (1:300) (Jackson Immunoresearch).

Sections were analysed using images captured with Zeiss Axiovision 2.05 (Imaging Associates, Thame, UK) and quantitation was performed using Openlab 2.2.1 software (Improvision, Viscount Centre II, Coventry, UK). Areas of muscle-specific staining were calculated by outlining the region of staining on the computer and determining the area within the trace. The area of the injected side was divided by the area of the uninjected side to yield a ratio. Sections throughout the region of the embryo expressing β-gal were randomly selected (from 10 sections per embryo in four embryos in the experiment shown) in order to reduce artifactual or biasing differences.

RESULTS

Expression of Hes6 in developing mouse muscle

Hes6 was cloned by searches of the mouse expressed sequence tag database for cDNAs encoding homologues of the *Drosophila* Enhancer of Split proteins that are expressed in muscle-derived libraries. We analysed the expression of Hes6 mRNA in mouse embryos using in situ hybridisation. Expression was detected in the developing brain and retina (data not shown), and in the developing somites in day 13 p.c. embryos as previously reported (Fig. 1A,B) (Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Vasiliauskas and Stern, 2000). In addition, we saw expression in developing skeletal muscle, both in the limbs and in the axial musculature, such as the diaphragm and intervertebral muscles in day 16 p.c. embryos (Fig. 1C,D and data not shown). However Hes6 mRNA was undetectable by in situ hybridisation in adult skeletal muscle (Fig. 1E,F). Consistent with this observation, analysis of adult mouse tissue by northern blotting revealed Hes6 mRNA in heart, liver, kidney and lung but not skeletal muscle (Fig. 1G). We went on to see if *Hes6* was expressed in the C2C12 mouse myoblast cell line when induced to differentiate by serum deprivation. In cells in high serum growth medium (GM), Hes6 mRNA was undetectable (Fig. 1H). However, when C2C12 cells were induced to differentiate in low serum differentiation medium (DM), transcription of *Hes6* was increased. The increase in *Hes6* mRNA paralleled the induction of myogenin transcription, confirming induction

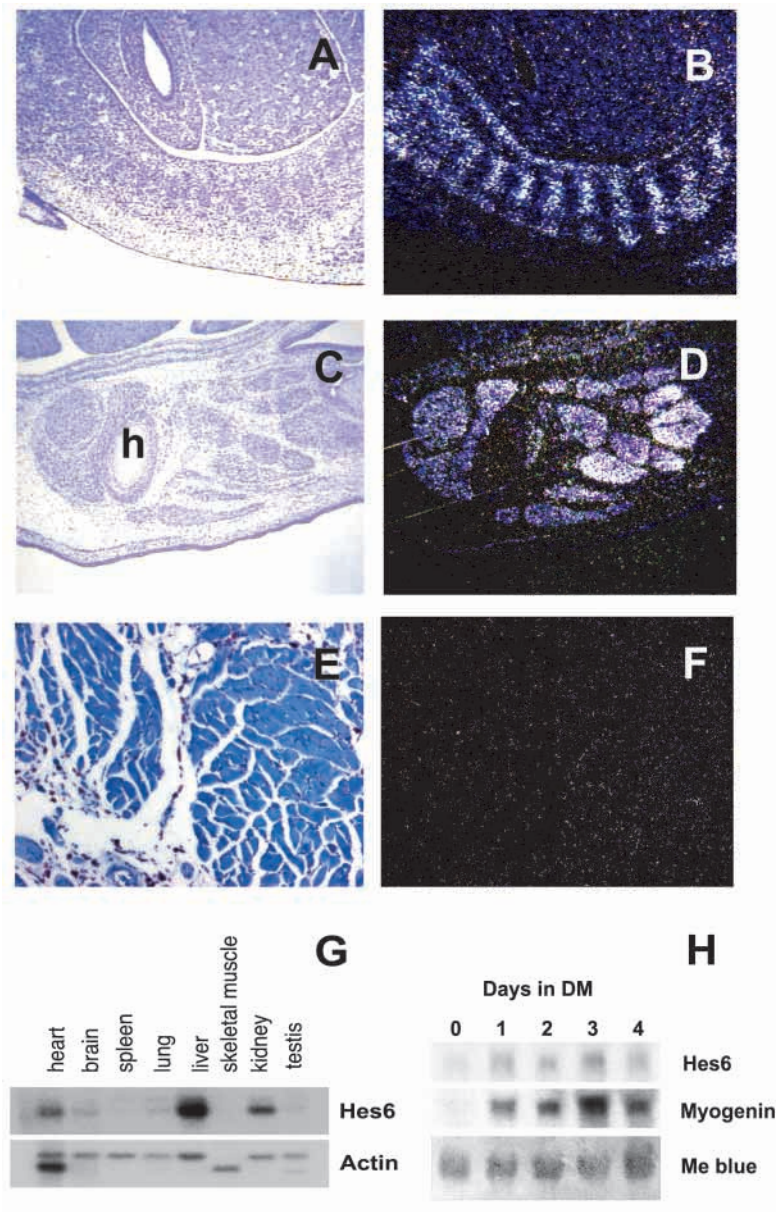

Fig. 1. *Hes6* transcription is detectable in developing muscle in mouse embryos and C2C12 myoblasts in culture. ^{35}S-labelled in situ hybridisation for *Hes6* mRNA on parasaggital sections of mouse embryos. (A,B) Day 13 p.c. embryo, *Hes6* mRNA is present in the developing somites. (C,D) Day 16 p.c. embryo. *Hes6* mRNA is expressed in the developing muscles in the forelimb; h, humerus. (E,F) Skeletal muscle (panniculus carnosus) from an adult mouse. *Hes6* mRNA is undetectable. (G) Northern blot analysis of *Hes6* expression in adult mouse tissues, compared with a γ actin control probe that also reacts with β actin. The difference in the actin hybridisation in the heart and skeletal muscle lanes reflects tissue specific differences in the levels of γ and β actin. Each lane was loaded with 2 μg of mRNA. *Hes6* mRNA runs at 1.4 kb. (H) *Hes6* transcription during C2C12 myoblast differentiation in vitro. C2C12 myoblasts were placed into differentiation medium for 0-4 days. RNA was isolated and analysed by northern blot. Each lane was loaded with 20 μg total RNA. The blot was also probed for myogenin, to confirm induction of differentiation and stained with Methylene Blue (Me blue) to reveal 18 S RNA. Result shown is typical of three independent experiments.

of differentiation (Andres and Walsh, 1996). Thus, *Hes6* is expressed during muscle differentiation in vivo and in vitro, but expression is downregulated in adult muscle.

Hes6 binds to an Enhancer of Split E box and mediates transcriptional repression

Hes6 has been shown to act indirectly by binding other orange domain-containing proteins, rather than by acting as a DNA-binding transcription factor (Bae et al., 2000; Koyano-Nakagawa et al., 2000). As reported previously, we found that Hes6 did not bind to the E and N box motifs recognised by other Hes proteins in an electrophoretic mobility shift assay (EMSA, data not shown) (Bae et al., 2000; Koyano-Nakagawa et al., 2000). However, we also tested binding to the Enhancer of Split E box (ESE box), a 12 nucleotide motif containing an E box identified by random oligonucleotide selection experiments with *Drosophila* EoS proteins (Jennings et al., 1999). Using in vitro translated protein, both wild-type and epitope-tagged Hes6 were found to bind an ESE box containing oligonucleotide in EMSA (Fig. 2A; data not shown). Binding was competed out by unlabelled oligonucleotide and a supershift of epitope tagged murine Hes6 demonstrated the specificity of the interaction. An excess of unlabelled E or N box-containing oligonucleotides had no effect on the binding of murine Hes6 to an ESE box, even in 200-fold molar excess of competitor (Fig. 2B). This confirms that the affinity of Hes6 was far higher for the ESE box than an E box or an N box.

Other Hes proteins show transcriptional repressor activity in transient transfection assays (Bessho et al., 2001; Sasai et al., 1992). We tested the ability of Hes6 to alter transcription of a model reporter containing ESE repeats. A fragment of the collagenase promoter (COL) that contains no E, N or ESE motifs was used for these experiments (Angel et al., 1987). When a control construct consisting of the COL promoter driving expression of luciferase was transfected into COS cells with Hes6 or β-galactosidase (β-gal)-encoding expression plasmids, there was no difference in transcription as assessed by luciferase activity (Fig. 2C). However, when two ESE box motifs were introduced 5′ to the COL promoter, there was a decrease in luciferase activity on transfection of murine Hes6 compared with β-gal in both COS and 3T3 cells (Fig. 2C; data not shown). The ratios in luciferase activity, Hes6:β-gal, in COS and 3T3 cells were 0.57:1 and 0.65:1 (means of four and three experiments, respectively, P=0.010 and P=0.0041, respectively, by two-tailed paired t-test). These data indicate that Hes6 represses transcription at a model promoter containing ESE boxes. The degree of transcriptional inhibition seen with Hes6 is similar to that described for other Hes family members such as Hes1 in similar reporter assays (Bae et al., 2000).

Effects of Hes6 overexpression on C2C12 myoblast differentiation in vitro

The dynamic expression of *Hes6* in mouse muscle and the

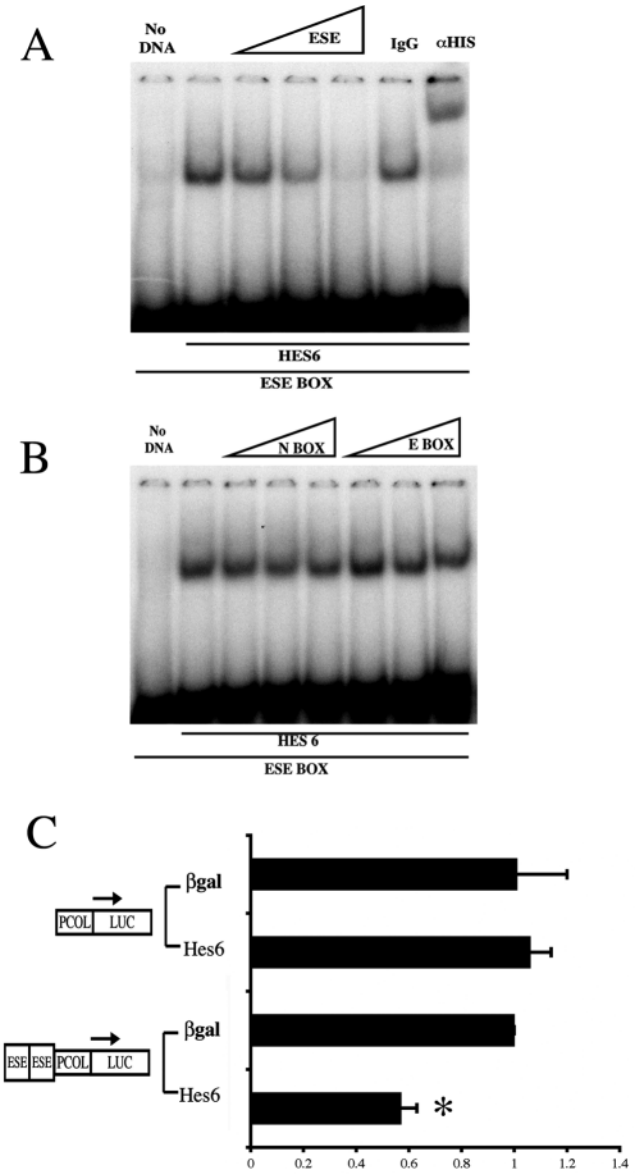

Fig. 2. Hes6 protein binds to an Enhancer of Split E (ESE) box and represses transcription at an ESE box containing promoter. EMSA of Hes6. In vitro translated protein was incubated with the oligonucleotides shown, as described in the Materials and Methods. (A) In vitro translation reactions containing either no DNA (left hand lane) or cDNA encoding Hes6 protein with an N terminal His Tag (remaining lanes) were incubated with a ^{32}P-labelled ESE box containing oligonucleotide. The binding reactions were carried out in the presence of unlabelled ESE box containing oligonucleotide present in 5-, 50- and 200-fold excess in the lanes shown. IgG indicates binding reaction carried out in the presence of control IgG; αHIS, binding reaction in the presence of an anti-HIS tag antibody. (B) In vitro translations with no DNA or Hes6 cDNA were incubated with a ^{32}P-labelled ESE box oligonucleotide, as in A. Unlabelled E box and N box competitor oligonucleotides, present in 5-, 50- and 200-fold excess were added to the reaction as shown. (C) COS cells were transiently transfected with pIRES2-EGFP plasmids with inserts encoding β-galactosidase or mouse Hes6, with a reporter vector consisting of a collagenase promoter driving expression of firefly luciferase (pCOLluc3) or pCOLluc3 with 2 ESE box motifs cloned immediately 5′ of the collagenase promoter (pCOLluc3-ESE). A *renilla* luciferase reporter (pRL-TK) was used as a control for transfection efficiency. The values shown represent the means of four independent experiments, each performed in triplicate or quadruplicate wells, normalised to the pIRES β-gal + pCOLluc3-ESE control. Error bars show s.e.m. **P*=0.010 using a two-tailed paired *t*-test, comparing Hes6 with β-gal control, when each was co-transfected with pCOLLuc3-ESE reporter.

induction of Hes6 expression on C2C12 differentiation suggested it might have a role in myogenesis. We set out to determine the effect of overexpression of mouse Hes6 (murine Hes6) on C2C12 myoblast differentiation in vitro. C2C12 myoblast differentiation follows an ordered sequence of molecular and cellular events. Myoblasts withdraw from the cell cycle coincident with induction of the cyclin-dependent kinase inhibitor (CDKI) p21^{Cip1} (Andres and Walsh, 1996). Subsequently, post-mitotic myoblasts undergo fusion to form myotubes that express terminal differentiation markers such as Troponin-T (Andres and Walsh, 1996).

A retroviral vector (pBabepuro) with puromycin selection was used to introduce Hes6 constructs to minimise the amount of cell culture required after transduction (Fig. 3A) (Morgenstern and Land, 1991). This approach avoids prolonged culture of C2C12 cells that can cause them to lose the ability to differentiate (Tachibana and Hemler, 1999). In each experiment, all cells were matched for passage number. A bicistronic EGFP vector was used to enable retrovirally derived protein expression in infected cells to be followed during differentiation without having to use an epitope tagged form of Hes6 (Fig. 3A). After selection in puromycin for 1-2 weeks, the proportion of cells expressing EFGP was assessed by flow cytometry. The proportion of cells expressing detectable levels of EGFP was 89% for β-gal, 95% for murine Hes6 and 94% for murine Hes6DBM (data not shown). To test if the DNA-binding activity of Hes6 was required for any changes in myogenic differentiation consequent on Hes6 overexpression, we mutated the DNA-binding site of murine Hes6 as shown in Table 1, to create Hes6DBM. As a control we used a virus encoding β-gal.

We found no apparent change in C2C12 myoblasts after infection with the Hes6 or Hes6DBM viruses compared with the control β-gal virus while the cells were maintained in GM (data not shown). Levels of GFP expression, assessed by flow cytometry, were similar with each virus (data not shown). However, when the cells were induced to differentiate by culture in DM for 5 days, a marked difference in cellular morphology was seen comparing either the Hes6 or Hes6DBM transduced cells with the control cells (Fig. 3B-D). Myotubes formed in cells transfected with all three constructs, but appeared elongated and narrowed with Hes6 or Hes6DBM compared with the β-gal control. No difference was seen comparing the morphology of murine Hes6 with murine Hes6DBM overexpressing cells.

Immunofluorescent staining revealed that Troponin-T-expressing myotubes were present in differentiated mouse Hes6 or mouse Hes6DBM overexpressing cultures as well as β-gal controls, though the number of nuclei per Troponin-T positive cell was reduced in the cells expressing the Hes6 constructs (Fig. 3E-G). The proportion of nuclei in Troponin-T-positive cells was typically 50% lower in murine Hes6 and murine Hes6 cultures than in β-gal control cultures (Fig. 4A). The ability of 20% of cells to fuse to form myotubes despite

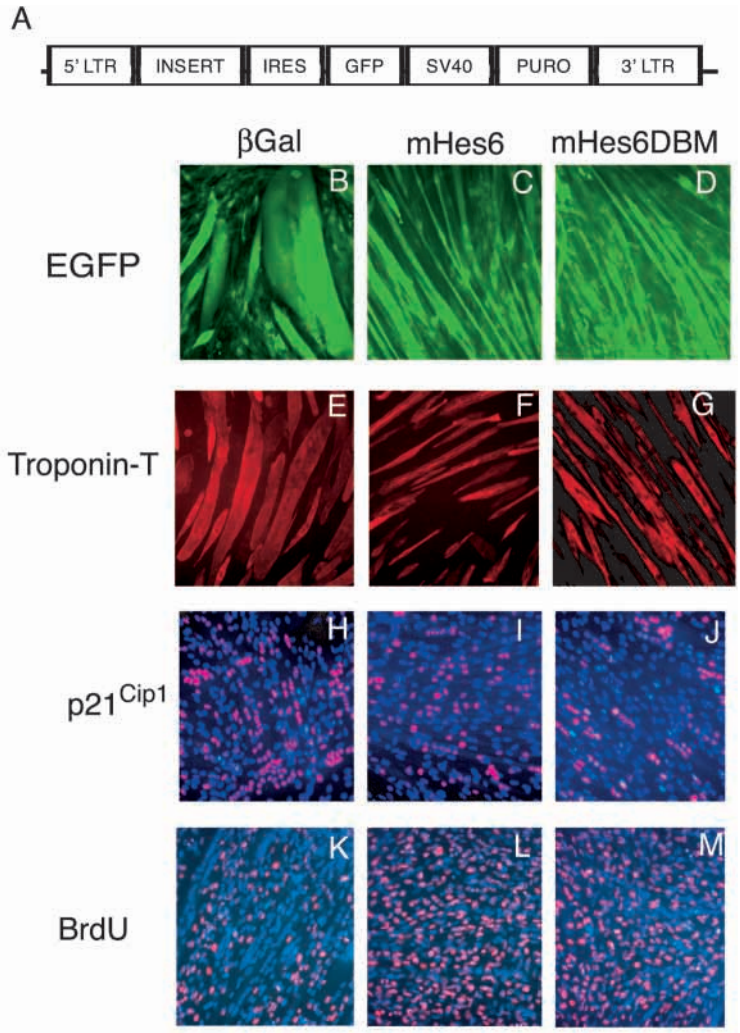

Fig. 3. Analysis of effects of overexpression of murine Hes6 and Hes6DBM on C2C12 myoblast differentiation. C2C12 myoblasts were infected with bicistronic retroviral vectors encoding β galactosidase (β-gal), murine Hes6 (mHes6) or Hes6DBM (mHes6DBM) and GFP as described in the Materials and Methods. Cells were cultured for 5 days in DM and then analysed by immunofluorescence. (A) Structure of retroviral vector. A bicistonic retroviral vector was used. Transcription of RNA encoding the insert, an internal ribosome entry site (IRES) and cDNA encoding EGFP is driven by the 5′ long terminal repeats (LTR). Translation of the bicistronic RNA produces two proteins, the inserted protein and EGFP. Expression of the puromycin resistance gene (puro) is driven by the SV40 viral promoter (SV40). (B-D) EGFP expression. Unstained cells were examined for EGFP fluorescence to confirm translation of the retrovirally expressed bicistronic RNA. There was no apparent difference in the level of EGFP expression, but in cells overexpressing Hes6 or Hes6DBM, the myotubes were elongated and narrower than β-gal-expressing cells. Appearances shown are typical of five independent experiments. (E-G) Troponin-T expression. Cells were stained with an anti-Troponin-T antibody. Myotubes expressed Troponin-T, a marker of terminal differentiation. The different morphology of myotubes in murine Hes6 and Hes6DBM cultures is seen; the number of nuclei per myotube is lower in murine Hes6 and Hes6DBM transduced cells compared with β-gal expressing cells. Appearances shown are typical of five independent experiments. (H-J) $p21^{Cip1}$ expression. Cells were stained with an anti-$p21^{Cip1}$ antibody, disclosed with a Cy3-conjugated secondary antibody. Nuclei were stained with DAPI. Rows of $p21^{Cip1}$-positive nuclei correspond to multinucleate myotubes. Fewer $p21^{Cip1}$-positive nuclei occur in the murine Hes6- and HES6DBM-expressing cells (see Fig. 4). (K-M) BrdU labelling to detect cells capable of re-entering into the cell cycle. To determine the proportion of cells in each culture that had undergone irreversible cell cycle withdrawal, cells were exposed to GM containing 50 μM BrdU for 20-22 hours after 5 days in DM. Nuclei were then stained with an anti-BrdU antibody and disclosed with a Cy3-conjugated secondary antibody. Nuclei were then stained with DAPI. More BrdU-positive cells are found in the murine Hes6- and Hes6DBM-expressing cells (see Fig. 4).

overexpression of Hes6 may reflect the heterogeneity of cell types in the C2C12 cell line and differences in the levels of Hes6 expression in the polyclonal population (Yoshida et al., 1998).

We hypothesised that the reduction in the number of cells undergoing fusion to form Troponin-T positive myotubes may reflect either a decrease in the number of myoblasts undergoing cell cycle withdrawal or a block in the process of cell fusion by post-mitotic myoblasts. To investigate if there was a decrease in the number of cells undergoing cell cycle withdrawal, cultures were examined for expression of the $p21^{Cip1}$ protein (Andres and Walsh, 1996). In western blots, the anti-$p21^{Cip1}$ antibody used recognised a protein corresponding in size to $p21^{Cip1}$ in β-gal, murine Hes6 and Hes6DBM-infected cells cultured in DM (data not shown). For cultures in GM, the proportion of $p21^{Cip1}$-positive nuclei was under 1% (data not shown). Control β-gal cultures significantly induced expression of $p21^{Cip1}$ within 48 hours of being placed in DM. By contrast, $p21^{Cip1}$ induction was markedly inhibited in cells overexpressing murine Hes6 or Hes6DBM (Fig. 3H-J; Fig. 4B). The induction of $p21^{Cip1}$ was not merely delayed, as the difference in expression between the murine Hes6 or Hes6DBM overexpressing cells and controls was maintained in cultures examined after 5 days in DM (Fig. 4B). There was no significant difference between Hes6- and Hes6DBM-expressing cells, suggesting that the binding of DNA by Hes6 is not required to inhibit induction of $p21^{Cip1}$.

Multiple CDKIs are active in myogenic differentiation in addition to $p21^{Cip1}$ (Franklin and Xiong, 1996; Zabludoff et al., 1998). We therefore needed to confirm that the cells that were $p21^{Cip1}$ negative were able to re-enter the cell cycle and were not post-mitotic because of the activity of other CDKIs. This was investigated by BrdU labelling of differentiated cultures. After 5 days in DM, cultures were transferred into GM containing BrdU for 20-22 hours (Andres and Walsh, 1996). The number of BrdU-positive cells was then analysed by immunofluorescence (Fig. 3K-M; Fig. 4C). The proportion of BrdU-positive nuclei was increased in cells expressing murine Hes6 or Hes6DBM compared with the control cells (Fig. 4C). In four experiments, the mean ratio of BrdU-positive cells (murine Hes6:βgal) was 1.45:1 (±0.07, s.e.m.) for murine Hes6 and 1.42:1 (±0.07, s.e.m.) for Hes6DBM, demonstrating no

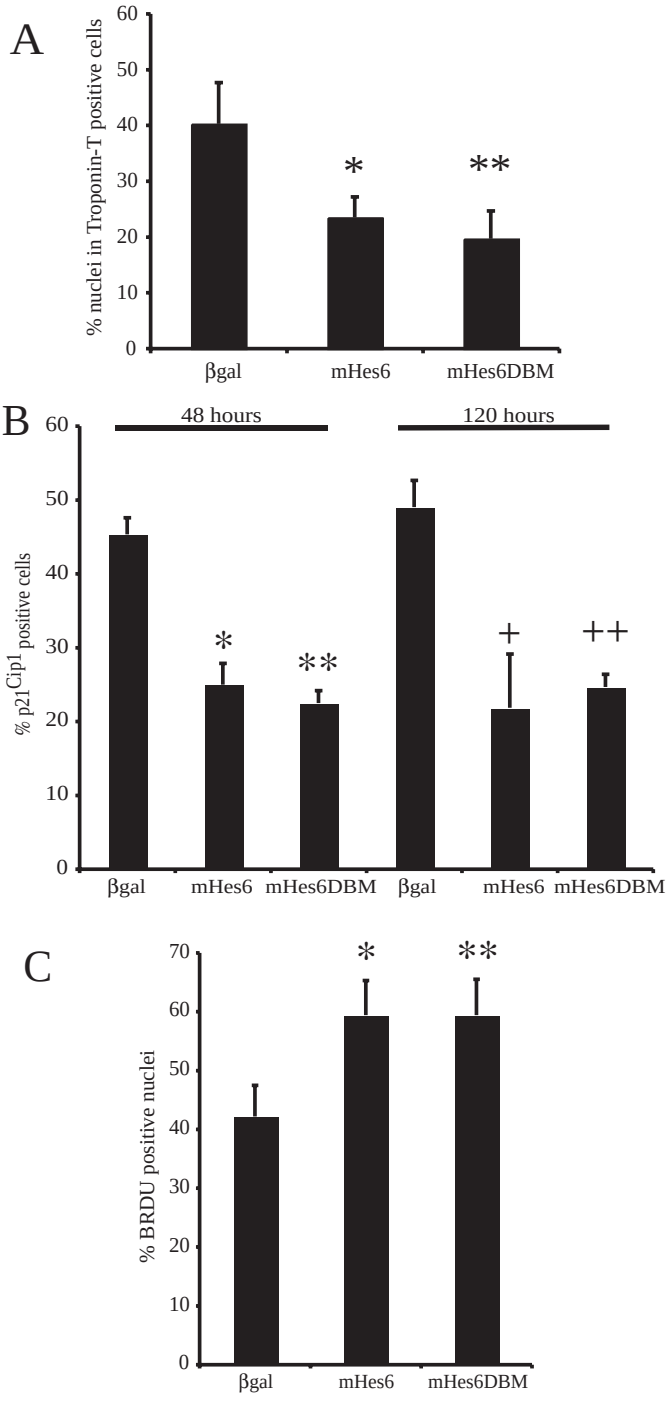

Fig. 4. Overexpression of murine Hes6 (mHes6) and Hes6DBM (mHes6DBM) in C2C12 myoblasts decreases the number of cells undergoing irreversible cell cycle withdrawal. (A) C2C12 cells were cultured in DM for 120 hours, fixed and stained for immunofluorescent analysis of the proportion of nuclei in Troponin-T-positive cells, as shown in Fig. 3. Results of a typical experiment are shown. The mean of five random fields containing a total of at least 600 nuclei is shown, error bars indicate s.d. **P*=0.004, ***P*=0.001, comparing murine Hes6 and Hes6DBM, respectively, with β-gal control using a two-tailed unpaired *t*-test. There was no significant difference between murine Hes6 and Hes6DBM. (B) C2C12 cells were cultured in DM for 48 or 120 hours, fixed and stained for immunofluorescent analysis of the proportion of $p21^{Cip1}$-positive nuclei as shown in Fig. 3. The mean proportion of $p21^{Cip1}$-positive nuclei expressed as a percentage of the total number nuclei in three independent experiments is shown. At least 1000 nuclei for each cell type were counted from random microscope fields at each time point in each experiment. Error bars show s.e.m. **P*=0.037, ***P*=0.040, comparing murine Hes6 with β-gal control using a two-tailed paired *t*-test at 48 and 120 hours, respectively. ^{+}P=0.013, ^{++}P=0.009, comparing murine Hes6DBM with β-gal control using a two-tailed paired *t*-test at 48 and 120 hours, respectively. There was no significant difference between the percentage of $p21^{Cip1}$-positive cells for murine Hes6 and Hes6DBM at either time point. (C) Cells were cultured in DM for 5 days and then placed in GM containing 50 μM BrdU for 20-22 hours. Cells were then fixed and stained for BrdU to determine the proportion of BrdU-positive nuclei as shown in Fig. 3. The mean proportion of BrdU-positive nuclei expressed as a percentage of the total number nuclei in three independent experiments is shown. At least 1000 nuclei for each cell type were counted from random microscope fields at each time point in each experiment. Error bars show s.e.m. **P*=0.008, ***P*=0.015, comparing murine Hes6 and Hes6DBM, respectively, with β-gal control using a two-tailed paired *t*-test. There was no significant difference between murine Hes6 and Hes6DBM.

difference between murine Hes6 and Hes6DBM. These observations indicate that the lack of large multinucleate myotubes in cultures overexpressing murine Hes6 or Hes6DBM is due, at least in part, to inhibition of myoblast terminal differentiation at or before the induction of $p21^{Cip1}$, resulting in a decrease in the number of post-mitotic myoblasts.

We have shown that Hes6 is expressed in the embryonic mouse myotome but expression is lost in adult muscle. In mouse myoblast cultures, sustained high level expression of Hes6 resulted in a block in terminal differentiation. These data support the hypothesis that downregulation of *Hes6* expression is required for terminal muscle differentiation.

Hes6 expression in *Xenopus* embryos

The in vitro data and expression pattern of *Hes6* in the embryonic myotome of mice supported the hypothesis that Hes6 has a role in myogenic differentiation. *Hes6* is highly conserved between mouse and *Xenopus*, suggesting *Xenopus* embryos as an appropriate system to test the effects of Hes6 overexpression on myogenesis in vivo. We examined the spatial expression pattern of *XHes6* in early gastrula and tailbud stage embryos using whole-mount in situ hybridisation. At early gastrula stages, *XHes6* was expressed in a ring around the closing blastopore but was excluded from the most dorsal region (Fig. 5A). This expression pattern matches that seen for *MyoD* which is expressed in involuting mesoderm that will mostly go on to differentiate into muscle (Fig. 5B) (Frank and Harland, 1991). However expression of *XHes6* is markedly different from MyoD at the tailbud stage. While MyoD expression is maintained in the myotome, *XHes6* expression is absent except for two to three chevron stripes immediately anterior to the tailbud and the tailbud itself (Fig. 5C,D) (Koyano-Nakagawa et al., 2000). The absence of XHes6 staining in the bulk of the differentiated myotome at tailbud stages is consistent with the model suggested by our in vitro experiments, that *Hes6* expression must be downregulated to allow terminal differentiation to proceed. Moreover, expression in chevrons anterior to the tailbud and in the tailbud itself is very reminiscent of the expression patterns of *XDelta2, Thylacine-1, ESR-4* and *ESR-5* which have all been implicated

Table 1. Hes6 constructs

Construct	Structure	Basic region
Murine Hes6	bHLH — ORANGE — WRPW	VEKKRRARIN
XHes6	bHLH — ORANGE — WRPW	VEKRRRARIN
Murine Hes6DBM	bHLH — ORANGE — WRPW	VKEEEDAEIN
XHes6DBM	bHLH — ORANGE — WRPW	VREREEADID
XHes6ΔWRPW	bHLH — ORANGE	VEKRRRARIN

Hes6 contains a basic helix-loop-helix domain (bHLH), an orange domain and a terminal WRPW motif, which binds to the transcriptional repressor Groucho and its homologues. In mouse Hes6DBM, the basic region of the bHLH domain was mutated as described in the Materials and Methods. *Xenopus* Hes6 and XHes6DBM constructs have been described previously (Koyano-Nakagawa et al., 2000). The amino acid sequence of the corresponding sequences of the basic domain in the normal and mutated constructs is shown. The XHes6ΔWRPW construct lacks the terminal WRPW motif.

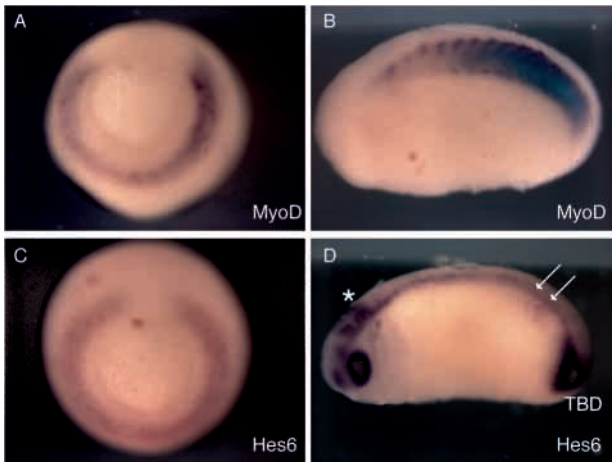

Fig. 5. *XHes6* is expressed in the presomitic mesoderm and somitic chevrons. Embryos were analysed by whole-mount in situ hybridisation at early gastrula stage (stage 10.5) (A,C) and tailbud stage (stage 22) (B,D) for expression of MyoD (A,B) and Hes6 (C,D). Both MyoD and Hes6 are found in a ring of prospective mesoderm around the dorsal pore at stage 10.5 (A,C, respectively). At stage 22, MyoD is restricted to myotome but is expressed uniformly throughout (B). Hes6 is found in the eye, brain (asterisk), neural tube, tailbud domain (TBD) and in two to three chevrons anterior to the TBD (arrows, D).

in the process of somitogenesis (Jen et al., 1999; Jen et al., 1997; Sparrow et al., 1998).

Hes6 overexpression enlarges the *Xenopus* myotome

To investigate the effect of Hes6 overexpression in vivo, we prepared RNA and injected it into one cell of two-cell stage embryos, with the lineage tracer β-gal. This procedure provides a useful internal control, as the first cleavage, which bisects the embryo into left and right, results in expression of message on only one side of the embryo. Three constructs were used to investigate the effect of XHes6 in vivo: (1) full-length wild-type XHes6, (2) a DNA-binding mutant, XHes6DBM, and (3) a mutant with a four amino acid deletion that disrupts binding to Groucho homologues (XHes6ΔWRPW) (see Table 1) (Koyano-Nakagawa et al., 2000). To confirm the activity of these constructs, embryos were assayed for the formation of primary neurones by staining for neural βtubulin (Nβtub) at the neural plate stage. All three constructs significantly upregulated Nβtub expression as previously reported (Fig. 6A; Table 2) (Koyano-Nakagawa et al., 2000).

To investigate whether XHes6 affected myotome formation in vivo, in situ hybridisation for MyoD and muscle actin (MA) mRNA were performed on overexpressing embryos at neural plate stages. While XHes6 and XHes6DBM both considerably enlarged the area of MyoD (Fig. 6D,E) and MA (Fig. 6G,H) expression, the XHes6ΔWRPW mutant had very little effect on either MyoD or MA transcripts (Fig. 6F,H; Table 2).

To quantify the effects of XHes6, XHes6DBM and XHes6ΔWRPW on myotome size, embryos were injected with synthetic message into one cell at the two-cell stage, fixed at stage 22, transversely sectioned and stained for expression of the early muscle marker, muscle actin (Fig. 7A-C). The cross-sectional area staining for muscle actin was calculated to give a measure of the size of the myotome. Three independent experiments yielded similar results; the results from one typical experiment are shown in Fig. 7D. Injection of wild-type XHes6 led to significant expansion of the size of the myotome on the injected versus the uninjected side (Fig. 7A,J). Interestingly, XHes6DBM also led to a significant increase in the size of the myotome, indicating that DNA binding is not essential for this effect (Fig. 7B,J). RNA made from the murine Hes6 and Hes6DBM constructs used in the in vitro experiments, also produced myotome expansion on overexpression in *Xenopus* (data not shown). However, overexpression of the XHes6Hes6ΔWRPW mutant did not lead to expansion of the myotome, indicating that protein-protein interactions mediated by the WRPW domain may be important in mediating myotome expansion (Fig. 7C,J). With all constructs, there were no significant changes in the size of the neural tube or the morphology of the epidermis, indicating the effects seen were specific to the myotome.

Table 2. Effects of XHes6 and mutants on expression of markers of neural and myogenic differentiation assessed by whole-mount in situ hybridisation

	Nβtub		MyoD		MA	
Injected constructs	Increase	No change	Increase	No change	Increase	No change
XHes6	41/66 (62%)	25/66 (38%)	48/62 (77%)	14/62 (23%)	32/52 (62%)	20/52 (38%)
XHes6DBM	43/62 (69%)	19/62 (31%)	35/52 (67%)	17/52 (33%)	38/53 (72%)	15/53 (28%)
XHes6ΔWRPW	31/69 (45%)	38/69 (55%)	5/38 (13%)	33/38 (87%)	9/51 (18%)	42/51 (82%)

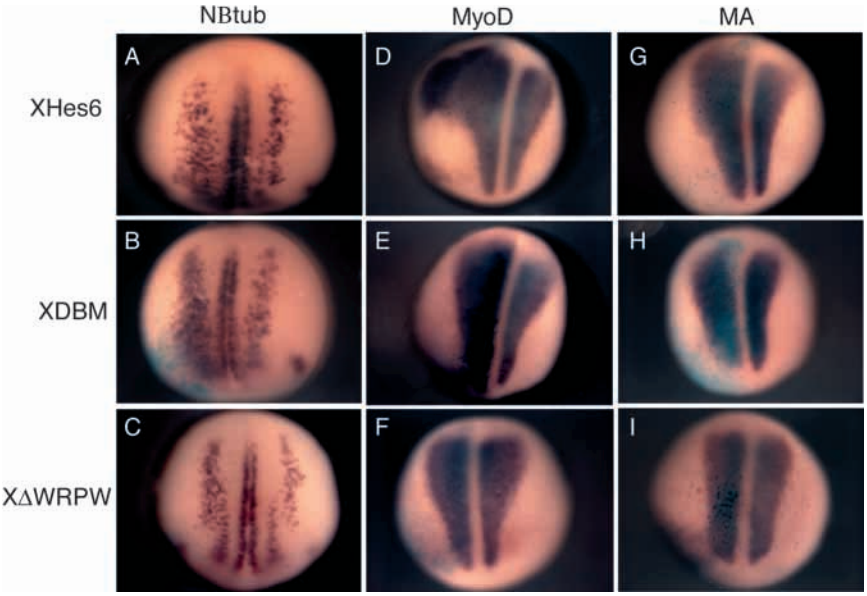

Fig. 6. XHes6 and XHes6DBM increase myotome size. Embryos were injected with 2 ng of XHes6 (A,D,G), XHes6DBM (B,E,H) or XHes6ΔWRPW (C,F,I) along with β-gal (light blue, injected side to left) and analysed at the neural plate stage for NβTub (A-C), MyoD (D-F) or α-sarcomeric actin (MA) (G-I) expression by whole-mount in situ hybridisation (purple). Overexpression of all three constructs upregulated NβTub expression (A-C), while only XHes6 (D,G) and XHes6DBM (E,H) upregulated the muscle markers MyoD and MA.

To confirm that the expanded myotome contained more cells, we counted the number of nuclei in longitudinal sections of the myotome double stained for nuclei and MA. At least 2500 nuclei were counted on nine or ten unselected sections of two typical embryos for each construct, from the same experiment as shown in Fig. 7. The ratio of cell number (injected/uninjected) was 3.3:1 (±0.46) for XHes6, 2.3:1 (±0.17) for XHes6DBM and 1.15:1 (±0.04) for XHes6ΔWRPW. All these differences were statistically significant (P<0.0011 by two-tailed t-test). Thus, XHes6 and XHes6DBM both cause a substantial increase in cell number within the myotome, but XHes6ΔWRPW has a minimal effect.

XHes6 overexpression inhibits terminal myogenic differentiation

Finally, our experiments with C2C12 cells have shown that

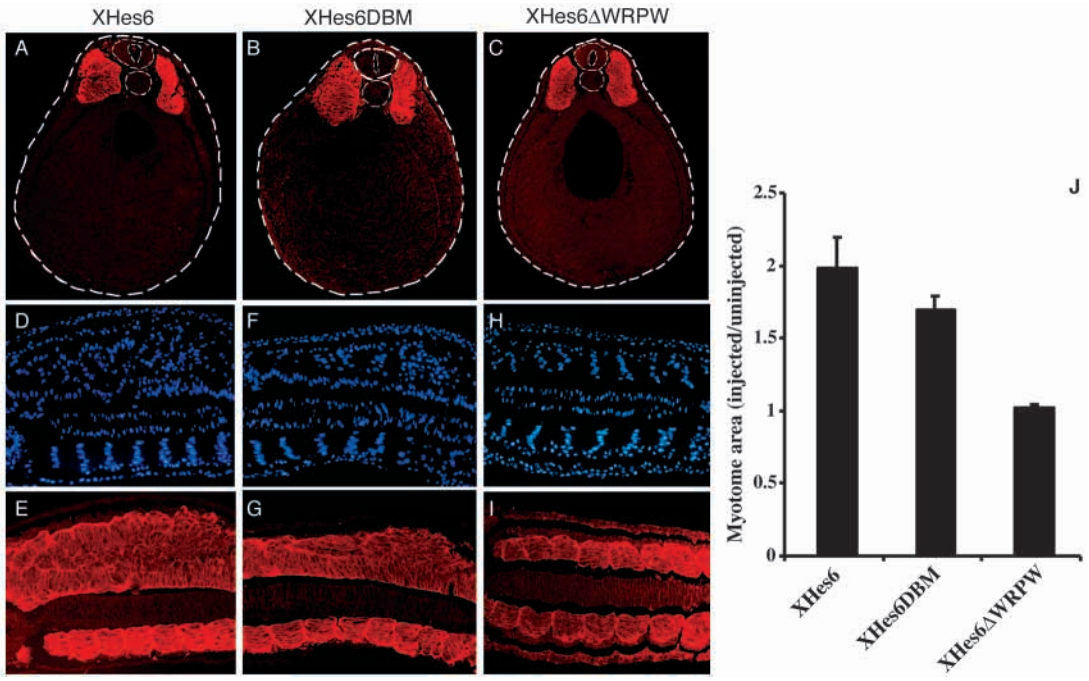

Fig. 7. XHes6 and XHes6DBM upregulate myogenesis and disrupt somitogenesis. Embryos were injected in one of two cells with 2 ng of (A,D,E) XHes6, (B,F,G) XHes6DBM or (C,H,I) XHes6ΔWRPW along with nuclear β-gal. At stage 22, the embryos were fixed and transversely (A-C) or longitudinally (D-I) sectioned and analysed for expression of MA (red); nuclei are stained with Hoechst (blue). Transverse sections are oriented with injected side to the left. Longitudinal sections are arranged with anterior towards the left and the injected side upwards. Broken white lines indicate the outlines of the embryo, the neural tube and the notochord. Quantitative image analysis was performed and the ratio of MA-expressing areas on injected and uninjected sides analysed as described in the Materials and Methods; results are shown in J. The error bars indicate the s.e.m. P values with a two-tailed t test, comparing mean ratios on injected and uninjected sides, were 0.017 for XHes6, 0.004 for XHes6DBM and 0.33 (not significant) for XHes6ΔWRPW. Both XHes6 and XHes6DBM cause complete disruption of somitogenesis (100% of embryos disrupted, n=26, 25 respectively), while XHes6ΔWRPW has a minimal effect (10% of embryos disrupted, n=20). In each embryo, the β-gal tracer was distributed both in the mesoderm and the ectoderm (data not shown)

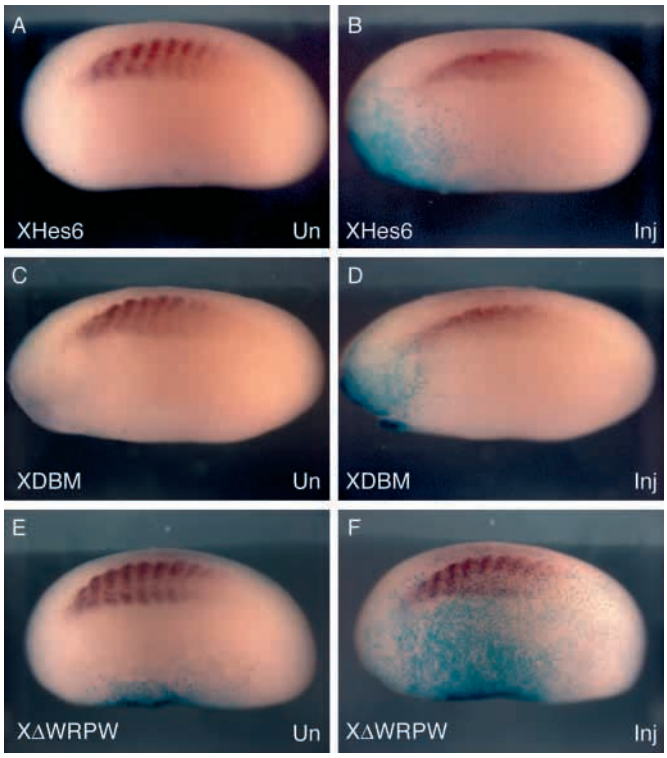

Fig. 8. XHes6 and XHes6DBM inhibit terminal myogenic differentiation. Embryos were injected in one of two cells with 2 ng of (A,B) XHes6, (C,D) XHes6DBM or (E,F) XHes6ΔWRPW with β-gal (light blue) and analysed at stage 22 for 12/101 expression (purple) by whole-mount antibody staining. XHes6 and XHes6DBM both decrease the area, intensity and pattern of 12/101 expression (compare A,C with B,D). However, XHes6ΔWRPW has little effect on the expression of this terminal myogenic differentiation marker (E,F).

overexpression of XHes6 can actively impede terminal differentiation of muscle cells. To see if overexpression of XHes6 has this effect in vivo, RNAs were injected into one cell of two-cell stage embryos along with the tracer β-gal. Embryos were allowed to develop until stage 22, fixed and stained with the antibody 12/101, a marker of terminal muscle differentiation (Kintner and Brockes, 1984). Strikingly, while the undifferentiated myotome was expanded in embryos injected with both XHes6 and XHes6DBM, the area and intensity of muscle staining for the 12/101 antibody on the injected compared to the uninjected side was decreased in 63% (19/30) of embryos overexpressing *XHes6* (Fig. 8A,B). This indicated an inhibition of terminal differentiation in the muscle on the injected side. Similar decreased staining was seen with the XHes6DBM construct where 89% (17/19) of embryos showed decreased 12/101 staining (Fig. 8C,D). By contrast, 12/101 staining was only decreased in 10% (4/41) embryos injected with the ΔWRPW construct. These results indicate that overexpression of XHes6 or a XHes6 DNA-binding mutant can inhibit terminal muscle differentiation in vivo, while a mutant lacking the WRPW domain cannot. In addition, gross observation of the 12/101 stained embryos indicated that the chevronated pattern, indicative of normal somite formation, was lost in 100% of embryos following both XHes6 and XHes6DBM overexpression (30/30 and 19/19 embryos, respectively). By contrast, very little difference was observable on overexpression of XHes6ΔWRPW; only 10% (4/41) of embryos had disruption of the staining pattern (Fig. 8E-F). These observations, raised the possibility that Hes6 was affecting somitogenesis.

XHes6 overexpression disrupts somitogenesis

Somitogenesis is a complex process whereby blocks of presomitic mesoderm, which originally lie randomly, align nuclei, segment and rotate through 90° so that the individual cells of the somite lie in parallel with the long axis of the embryo (Hamilton, 1969). We have investigated whether overexpression of XHes6 and its mutant derivatives disrupt this process. RNAs were again injected unilaterally into one cell of two-cell stage embryos and the embryos allowed to develop to tailbud stage. Embryos were sectioned longitudinally and stained with an anti-MA antibody to reveal somite morphology and Hoechst to reveal the nuclei. As well as resulting in a substantial increase in myotome size, as discussed above, overexpression of wild-type XHes6 also dramatically disrupted somitogenesis. Actin staining, which allows visualisation of gross myotomal morphology, revealed that individual somites had failed to segregate and essentially no intersomitic boundaries were visible (Fig. 7E). Moreover, Hoechst staining in the myotome revealed a failure of nuclei to line up or rotate (Fig. 7D). Similar disruption of somite morphology is seen on overexpression of XHes6DBM (Fig. 7F,G). By contrast, the somites formed essentially normally in XHes6ΔWRPW-injected embryos, indicating that protein-protein interactions involving the WRPW region were important for the disrupted somite phenotype of XHes6 (Fig. 7H,I).

DISCUSSION

We have presented evidence that Hes6 may have a role in regulating myogenic differentiation and somite formation. Hes6 is expressed in myogenic cells in vitro and in the embryonic myotome in mouse and *Xenopus*. Overexpression of Hes6 inhibits the terminal differentiation of C2C12 myoblasts, resulting in a decrease in the number of cells withdrawn from the cell cycle in differentiated cultures. Hes6 overexpression in *Xenopus* results in a complex phenotype, with an increase in size of the myotome, a decrease in terminal differentiation and a failure of somitogenesis. By using mutant forms of *Hes6* we have demonstrated that the DNA-binding activity of the protein is not required to produce these phenotypes in myoblasts in culture or in *Xenopus* embryos.

Function of Hes6 protein

As previously reported, Hes6 shows no affinity for E or N box containing oligonucleotides in EMSA assays (data not shown) (Bae et al., 2000; Koyano-Nakagawa et al., 2000). However, it does bind to the same ESE box as *Drosophila* EoS proteins and represses transcription at an ESE box-containing reporter (Jennings et al., 1999). Thus, Hes6 exhibits the properties of a negative regulator of transcription in in vitro reporter assays, like other Hes proteins, but the divergent structure of the DNA-binding domain of Hes6 confers different DNA-binding properties (Bessho et al., 2001; Sasai et al., 1992). However, the identical phenotypes seen with wild-type and DBM forms

of Hes6 in vitro and in vivo suggest that DNA binding by Hes6 is not required for its roles in myogenesis or somitogenesis.

The XHes6ΔWRPW mutant has a minimal effect on the size of the myotome, the expression of late markers of terminal muscle differentiation, and somitogenesis. This finding contrasts with the effect of this mutant on neurogenesis, where as shown here and reported previously it produces an increase in the proportion of tubulin-positive cells in about 50% of embryos (Fig. 6C; Table 2) (Koyano-Nakagawa et al., 2000). This suggests that the effects of XHes6 and XHes6DBM overexpression on myogenesis and somite formation require the recruitment of *Groucho* homologues or other proteins via the WRPW domain, whereas the neural phenotype does not. This is consistent with Hes6 having a role as a non-DNA-binding repressor of transcription in myogenic cells, although a quantitative difference in the effect of the WRPW mutant on muscle and nerve differentiation cannot be excluded.

The role of Hes6 in myogenesis in vitro

Overexpression of murine Hes6 or murine Hes6DBM in C2C12 myoblasts results in a decreased number of nuclei per myotube, which is likely to reflect a reduction in the number of cells expressing $p21^{Cip1}$ and undergoing irreversible cell cycle withdrawal. It is interesting to compare the results of expression of full-length Hes6 in C2C12 cells with recently reported data on the function of a truncated form of Hes6 ($Hes6^{T}$), which lacks the first 16 amino acids of full-length Hes6 (Gao et al., 2001). In contrast to full-length Hes6, $Hes6^{T}$ synergises with Hes1 in repression of an N box reporter, and promotes C2C12 terminal differentiation (Bae et al., 2000; Gao et al., 2001). This indicates an important function for the N terminus of the wild-type protein.

Protein-protein interactions seem to mediate the effect of Hes6 on C2C12 cells as Hes6DBM produces the same inhibition of differentiation as the wild-type protein. A candidate for such an interaction is Hes1, a Notch-induced member of the Hes family (Jarriault et al., 1998; Kuroda et al., 1999). Hes6 has been shown to interact with Hes1 in in vitro binding assays and in reporter assays, where Hes6 relieves Hes1-mediated inhibition of an N box reporter (Bae et al., 2000). *Hes1* overexpression causes a variety of responses in different cell lines. In differentiating PC12 cells, Hes1 parallels the effects of Hes6 overexpression that we observe in C2C12 cells. Acting via the orange domain, Hes1 inhibits the $p21^{Cip1}$ promoter when PC12 cells are induced to differentiate by nerve growth factor (Castella et al., 2000). However, Hes1 has no effect on cell cycle in a B cell line (Morimura et al., 2000). In contrast to Hes6, Hes1 overexpression does not block the differentiation of C2C12 cells, indicating that these proteins have distinct functions in myogenesis (Shawber et al., 1996). It is possible that some aspects of the phenotype seen in Hes6 and Hes6DBM over expressing cells are due to inhibition of Hes1 function by Hes6. Further investigation is required to see if additional interacting proteins are also involved.

Hes6 in *Xenopus* myogenesis

While C2C12 myoblasts are useful for studying late myogenic differentiation, their committed lineage prevents the modelling of early events. To study the role of Hes6 in both early and late myogenesis in vivo, we have taken the complimentary approach of overexpression in *Xenopus* embryos.

Analysis of the expression pattern of *XHes6* revealed that it is localised in lateral and ventral involuting mesoderm at the gastrula stage, overlapping the expression of the myogenic gene *MyoD*, consistent with a role in early myogenesis (Frank and Harland, 1991). However, by the tailbud stage, and in contrast to MyoD, XHes6 RNA is undetectable in anterior differentiated muscle. This indicates that *XHes6* is downregulated in terminal differentiation, mirroring the loss of murine Hes6 expression that occurs as the embryonic mouse myotome differentiates into adult skeletal muscle. However, *XHes6* expression is still conspicuously expressed in the tailbud where new muscle is being formed (Davis and Kirschner, 2000).

Strikingly, the phenotype produced by *XHes6* overexpression is significantly tissue restricted. There is expansion of the myotome and an increase in the number of differentiated primary neurones, but no gross expansion of the neural tube or hypertrophy of the epidermis (Fig. 6) (Koyano-Nakagawa et al., 2000). This lineage-specific expansion is in marked contrast to the phenotype produced by overexpression of the cytoplasmic domain of Notch, which expands the myotome, epidermis and neural tube (Coffman et al., 1993).

The expansion of the myotome and corresponding increase in cell number produced by XHes6 overexpression may be due to increased recruitment of cells into the muscle lineage and/or increased myoblast proliferation. Indeed early expansion of *MyoD* and *muscle actin* on *XHes*6 overexpression indicates a possible role in recruitment, while the repression of terminal muscle differentiation in vivo (Fig. 8) and downregulation $p21^{Cip1}$ in myoblasts in vitro (Fig. 4) implies it may also prolong proliferation. The increase in size and cell number in the *Xenopus* myotome may result from a combination of effects on recruitment and proliferation.

Interestingly, while *XHes6* overexpression promotes the formation of tissue-expressing markers of muscle commitment and early differentiation, it actually inhibits the expression of the terminal differentiation marker 12/101 in the enlarged myotome. The observation that XHes6 is downregulated in vivo in anterior muscle at the early tailbud stage is consistent with a requirement for *XHes6* expression to be lost before terminal differentiation can occur.

Our analysis of overexpression of *Hes6* mutants has shown that in *Xenopus* myogenesis, DNA binding by Hes6 is not required to produce a muscle phenotype, while protein-protein interactions mediated by the WRPW domain are essential. One class of proteins that interact with the WRPW domain are the homologues of *Drosophila* Groucho (Molenaar et al., 2000; Paroush et al., 1994). XHes6 has also been shown to bind other orange domain-containing proteins such as Xhairy1 and Xhairy2a, such interactions probably being mediated by the orange domain (Dawson et al., 1995; Koyano-Nakagawa et al., 2000). The proteins that interact with XHes6 to produce the phenotypes described here remain to be defined, but the lineage restricted nature of the effects of *XHes6* overexpression suggest that its interaction partners will be expressed in developing muscle and nerve but not other tissues.

Hes6 and somite formation

In tailbud stage embryos, *XHes6* is expressed in two to three chevrons immediately anterior to the tailbud (Fig. 5) (Koyano-Nakagawa et al., 2000). Very similar expression in chevrons is

seen in several genes implicated in somite segmentation, such as *ESR-4*, *ESR-5*, *Thalacine-1* and *XDelta-2* (Jen et al., 1999; Jen et al., 1997; Sparrow et al., 1998). Thus, *XHes6* is expressed in a spatial and temporal pattern suggestive of a role in somite formation. Overexpression of *XHes6* not only results in an expansion of the myotome but also causes substantial disruption of somite organisation; nuclei fail to align, cells fail to rotate and intersomite boundaries fail to form. Instead the myocytes remain in a chaotic mass. It is unlikely that the observed defect in somitogenesis is caused solely by an increase in the size of the myotome. Overexpression of *MyoD* results in a substantial increase in myotome size, yet only mild, if any, disruption of nuclear alignment, cell rotation and somite boundary formation occurs (Ludolph et al., 1994) (A. E. V. and A. P., unpublished). We favour the possibility that *XHes6* may play a role in somite formation beyond its ability to regulate myotome size and muscle differentiation. Analysis of Hes6 mutants again indicates that DNA-binding activity is not required to disrupt somitogenesis, but that the presence of the WRPW domain is essential.

Somite formation in *Xenopus* is regulated by Notch signalling, which controls the expression of the EoS homologues *ESR-4* and *ESR-5* (Jen et al., 1999). While *Hes6*, *ESR-4* and *ESR-5* have structural homology and are all found expressed in similar posterior chevrons corresponding to prospective somites, they have key functional differences. Overexpression of *ESR-5* results in a failure of somite formation similar to that seen with *XHes6*, yet it does not affect the size of the myotome or terminal muscle differentiation (Jen et al., 1999). Additionally, *XHes6* expression does not appear to be regulated by Notch signalling, unlike that of *ESR-4* and *ESR-5*, which both lie within the Notch pathway (Jen et al., 1999). As ESR-4 and ESR-5 both contain the Groucho homologue binding motif, WRPW, but have a phenotype distinct from Hes6, it is unlikely that the effect of Hes6 on the myotome is mediated by titration of Groucho homologues alone.

Hairy is a candidate effector of somitogenesis in *Xenopus*, zebrafish and chick (Jen et al., 1997; Jouve et al., 2000; Takke and Campos-Ortega, 1999). XHes6 has been shown to bind to both Xhairy1 and Xhairy2a in vitro and in vivo (Koyano-Nakagawa et al., 2000). Such heteromultimers may differ in their transcriptional properties from homomultimeric Hairy complexes, allowing Hes6 to regulate the transcriptional activity of Hairy (Bae et al., 2000). Hes6 may also regulate the level of *Hairy* expression as overexpression of *XHes6* or *XHes6DBM* increases transcription of *Xhairy1* in *Xenopus* animal caps (Koyano-Nakagawa et al., 2000). These observations raise the possibility that Hes6 may exert its effects on somite formation via effects on *Hairy* homologues; further investigation is required to see if this is indeed the case.

Thus, we have demonstrated that *Hes6* is expressed in developing muscle and regulates the differentiation of cultured myoblasts. In vivo overexpression of *XHes6* in *Xenopus* has revealed further roles in regulation of myotome size, terminal differentiation and somitogenesis. As DNA binding by Hes6 seems not to be essential, it will be interesting to determine which protein-protein interactions are required to mediate these multiple, tissue-specific effects.

We thank Dr Chris Kintner for the gift of *Xenopus* Hes6 constructs. J. C., Y. Z. and P. H. J. were supported by an Imperial Cancer Research Fund Clinician Scientist Fellowship, P. H. J. is supported by Cancer Research UK (formerly Cancer Research Campaign) Senior Clinical Fellowship, SP2564/0201. A. E. V. is supported by a Howard Hughes Medical Institute Predoctoral Fellowship. A. P. is supported by Project Grant number SP24776/0101, from Cancer Research UK. P. H. J. thanks Tariq Enver and Alan Ashworth at the Institute of Cancer Research, London, and Adrian Harris and Ian Hickson at the Weatherall Institute of Molecular Medicine, Oxford, for their support during this project.

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